# Central deficiency of norepinephrine synthesis and norepinephrinergic neurotransmission contributes to seizure-induced respiratory arrest

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ARTICLEINFO

*Keywords:*
Sudden unexpected death in epilepsy
Prazosin
Norepinephrine
Norepinephrine receptors
Tyrosine hydroxylase

ABSTRACT

Sudden unexpected death in epilepsy (SUDEP) is the leading cause of mortality in patients with intractable epilepsy. However, the pathogenesis of SUDEP seems to be poorly understood. Our previous findings showed that the incidence of seizure-induced respiratory arrest (S-IRA) was markedly reduced by atomoxetine in a murine SUDEP model. Because the central norepinephrine α-1 receptor (NEα-1R) plays a vital role in regulating respiratory function, we hypothesized that the suppression of S-IRA by atomoxetine was mediated by NE/NEα-1R interactions that can be reversed by NEα-1R antagonism. We examined whether atomoxetine-mediated suppression of S-IRA evoked by either acoustic stimulation or pentylenetetrazole (PTZ) in DBA/1 mice can be reversed by intraperitoneal (IP) and intracerebroventricular (ICV) administration of prazosin, a selective antagonist of NEα-1R. The content and activity of tyrosine hydroxylase (TH), a rate-limiting enzyme for NE synthesis, in the lower brainstem was measured by ELISA. Electroencephalograms (EEG) were obtained from using the PTZ-evoked SUDEP model. In our models, atomoxetine-mediated suppression of S-IRA evoked by either acoustic stimulation or PTZ was significantly reversed by low doses of IP and ICV prazosin. Neither repetitive acoustic stimulation nor S-IRA reduced TH levels in lower brainstem. However, the enzyme activity of TH levels in lower brainstem was significantly increased by mechanical ventilation with DBA/1 mice, which makes the dying DBA/1 mice suffering from S-IRA and SUDEP recover. EEG data showed that although the protective effect of atomoxetine was reversed by prazosin, neither drug suppressed EEG activity. These data suggest that deficient synthesis of NE and norepinephrinergic neurotransmission contributed to S-IRA and that the NEα-1R is a potential therapeutic target for the prevention of SUDEP.

## 1. Introduction

Sudden unexpected death in epilepsy (SUDEP) had been recognized as the leading cause for death of epilepsy patients, which accounts for up to 17 % of deaths in epilepsy patients, and in term of potential life loss due to SUDEP, ranks second only to the stroke [1–4]. Although the pathogenesis of SUDEP is unclear, multiple studies indicate that most cases are caused by cardiopulmonary dysfunction [5,6]. Our previous findings demonstrated that seizure-induced respiratory arrest (S-IRA) is the leading cause of SUDEP evoked by either acoustic stimulation or pentylenetetrazole (PTZ) in DBA/1 murine models, and was significantly relieved by atomoxetine, a selective norepinephrine (NE) reuptake inhibitor that is commonly used for treating attention-deficit hyperactivity disorder (ADHD) [7–10]. However, the mechanism underlying atomoxetine suppression of S-IRA remains unknown [9,10]. It is generally accepted that NE α-1 receptor (NEα-1R) plays a vital role in the central regulation of respiration [11–13]. Also reported is symptoms of DBA/1 SUDEP model imposed by audiogenic seizures or PTZ can simulate the generalized seizures of epilepsy patients [7,14], based on which we hypothesized and tried to test if NEα-1R modulates S-IRA and is a key therapeutic target to prevent SUDEP in these models.

To study the roles of NE and NEα-1R in the pathogenesis of S-IRA in DBA/1 mice from behavioral and electrophysiologic perspectives, we first attempted to enhance NE bioavailability in the central synaptic spaces by using atomoxetine. We then sought to determine whether the atomoxetine-mediated suppression of S-IRA can be reversed by the peripheral and central administration of prazosin, a selective NEα-1R antagonist. To exclude the possibility that neurologic effects of prazosin could be mediated by altered cardiovascular function, we measured the changes of mean systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) in DBA/1 mice from different dosage groups. Furthermore, we studied the effect of prazosin on atomoxetine-mediated suppression of S-IRA by

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https://doi.org/10.1016/j.biopha.2020.111024
Received 7 September 2020; Received in revised form 4 November 2020; Accepted 15 November 2020
Available online 21 November 2020
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evaluating seizure scores and electroencephalographic (EEG) recordings.

In our study, atomoxetine-mediated suppression of S-IRA following acoustic stimulation and PTZ injection in DBA/1 SUDEP mice models was significantly reversed by intraperitoneal (IP) and intracerebroventricular (ICV) injections of prazosin in two models, the observations of which were not accompanied with changes in seizure behavior, cardiovascular function, or EEG activity. In addition, our results provide the first evidence that the occurrence of S-IRA can be attributed to the decreased enzyme activity of TH in lower brainstem, which led to the lower synthesis of NE in brainstem. Thus, our results suggested that enhancing central the synthesis of NE and norepinephrinergic neurotransmission, and targeting the central NEα-1R may represent rational therapeutic strategies to prevent SUDEP.

## 2. Methods

### *2.1. Animals*

All experimental procedures were compliant with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Advisory Committee at Zhejiang University. DBA/1 mice were housed and bred in the Animal Center of Zhejiang University School of Medicine and provided with rodent food and water ad libitum. DBA/1 mice of either gender were used in the experiments, as previous study suggested that gender is not a variable affecting S-IRA in DBA/1 mice [15]. In the acoustic stimulation murine model, DBA/1 mice were “primed” starting from postnatal day 26–28 to establish consistent susceptibility to audiogenic seizures and S-IRA. Atomoxetine (dissolved in saline and given IP) and prazosin were administered 120 min. and 30 min. prior to acoustic stimulation, respectively. In the another model of PTZ-evoked seizure model, PTZ was administered to non-primed DBA/1 mice of approximately 8 weeks of age. The two models were established just as described previously [7–10] and it will be described in detail in the following section of seizure induction and resuscitation.

### *2.2. Seizure induction and resuscitation*

S-IRA was evoked by acoustic stimulation and intraperitoneal (IP) administration of PTZ, as previously described [7–10]. As for the acoustic stimulation model, each mouse was placed in a cylindrical plexiglass chamber in a sound-isolated room, and audiogenic seizures (AGSZs) were evoked by an electric bell (96 dB SPL, Zhejiang People’s Electronics, China). The acoustic stimulation was given for a maximum duration of 60 s or until the mouse exhibited tonic seizures and the extent of which culminated in tonic hindlimb extension and S-IRA in each group.. Mice with S-IRA were resuscitated within 5 s posterior to the final respiratory gasp using a rodent respirator (YanYu Instruments, ShangHai, China), which pumped room air by a volume of 1 mL at 180 strokes/min to induce visible displacement of the chest. As for the assessment of susceptibility to S-IRA, each primed DBA/1 mice was always reconfirmed 24 h before drug or vehicle administration. S-IRA was evoked in all non-primed DBA/1 mice by a single dose IP administration of PTZ (Cat # P6500; Sigma-Aldrich, St. Louis, MO, 75 mg/kg). The resuscitation parameter of PTZ injection model is the same to that of the acoustic stimulation model.

### *2.3. Effect of IP administration of prazosin on atomoxetine-mediated suppression of S-IRA*

A vehicle and different doses of prazosin (Cat # P7791; Sigma- Aldrich**)** were administered to different groups of mice which were then acousticaly stimulated. Susceptibility to S-IRA in primed DBA/1 mice was confirmed 24 h prior to treatment of atomoxetine(Ca # Y0001586; Sigma-Aldrich**)** or vehicle, which was followed by prazosin administration respectively. Specifically, atomoxetine (15 mg/kg) and prazosin (0.001− 0.1 mg/kg) were given by IP injection 120 min and 30 min prior to acoustic stimulation, respectively. In the vehicle control group, saline and 25 % dimethyl sulfoxide (DMSO) were given by IP injection 120 min and 30 min prior to acoustic stimulation respectively. The incidence of S-IRA, latency to AGSZs, duration of wild running, clonic seizures, tonic- clonic seizures, and seizure scores were videotaped for offline analysis [16,17] (n = 6–9/group).

### *2.4. Effect of IP administration of prazosin on blood pressures and heart rate*

Non-invasive measurements of heart rate and blood pressures at 37 °C were performed in conscious DBA/1 mice treated with either atomoxetine or vehicle using a tail-cuff system (BP-98A, Blood Pressure Analysis System, Softron, Japan). To ensure the accuracy and reproducibility, the mice were trained for 3 days prior to the experiment, and measurements were taken each day at the same time. In the control group, atomoxetine (15 mg/kg) and 25 % DMSO were given by IP injection 120 min and 30 min prior to acoustic stimulation, respectively. The experimental group was given atomoxetine (15 mg/kg) and prazosin (2 mg/kg dissolved in 25 % DMSO) by IP injection 120 min and 30 min prior to acoustic stimulation, respectively. According to the protocol of tail-cuff system, measurements of SBP, DBP, MAP and HR were measured during 60 s intervals at 120 min and 25 min before and 5 min after acoustic stimulation (n = 6–9/group). *2.5. Effect of ICV prazosin administration on S-IRA*

An ICV guide cannula(O.D.I.41 × I.D.O.0.25 mm/M3.5, 62,004, RWD Life Science Inc.,China)was implanted into the right lateral ventricle (AP – 0.45 mm; ML – 1.0 mm; V– 2.50 mm) to enable microinjection as previously described (Zhao, et al., 2017). The experimental groupings were: 1) Saline (IP) was administered 120 min prior to PTZ (75 mg/kg, IP) and 25 % DMSO at (2 μl, at a rate of 0.5 μl/min ICV) 15 min prior to PTZ injection as controls. 2) Atomoxetine (15 mg/kg, IP) was administered 120 min prior to

PTZ (75 mg/kg, IP) and 25 % DMSO (2 µl, at a rate of 0.5 µl/min ICV) 15 min prior to PTZ injection. 3) Atomoxetine (15 mg/kg, IP) was administered 120 min prior to PTZ (75 mg/kg, IP), and prazosin (4.764 nmol and 9.528 nmol, dissolved in 2 µl 25 % DMSO, at a rate of 0.5 µl/min ICV) 15 min prior to PTZ injection (n = 6–9/group).

### *2.6. Effect of ICV prazosin administration on EEG*

After the implantation of ICV guide cannulas described above, DBA/ 1 mice were implanted with a headstage for EEG recordings. The headstage consisted of a 6-pin connector soldered to 2 EEG screws. The EEG electrode was inserted into the surface of cerebral cortex fixed by screws inserted into the cranium with a layer of dental cement mixed with cyanoacrylate glue. EEG recordings were performed one week after the surgery. The experimental groups were divided into 3 groups as follows: 1) Saline (IP) and 25 % DMSO (2 µl, at a rate of 0.5 µl/min ICV) at 120 min and 15 min prior to PTZ (75 mg/kg, IP), respectively, as controls. 2) Atomoxetine (15 mg/kg, IP) and 25 % DMSO (2 µl, at a rate of 0.5 µl/min ICV) 120 min and 15 min prior to PTZ (75 mg/kg, IP), respectively. 3) Atomoxetine (15 mg/kg, IP) and prazosin (4.764 and 9.528 nmol dissolved in 2 µl 25 % DMSO administered a rate of 0.5 µl/ min ICV) 120 min and 15 min prior to PTZ (75 mg/kg, IP), respectively. EEG recording began immediately to after the completion of ICV injections. ICV placement was confirmed by histology with DAPI staining as previously described [10]. The time of recording EEG started 5 min. before PTZ injection and ended at 20 min after injection or upon death of mice. EEG data was recorded and 0− 100 Hz spectral power densities were calculated (deta, 0− 4 Hz; theta,4− 8 Hz; alpha,8− 12 Hz; beta, 12− 30 Hz; gamma, 30− 100 Hz) using Neuroscan System software package (Compumedics, Melbourne, Australia).

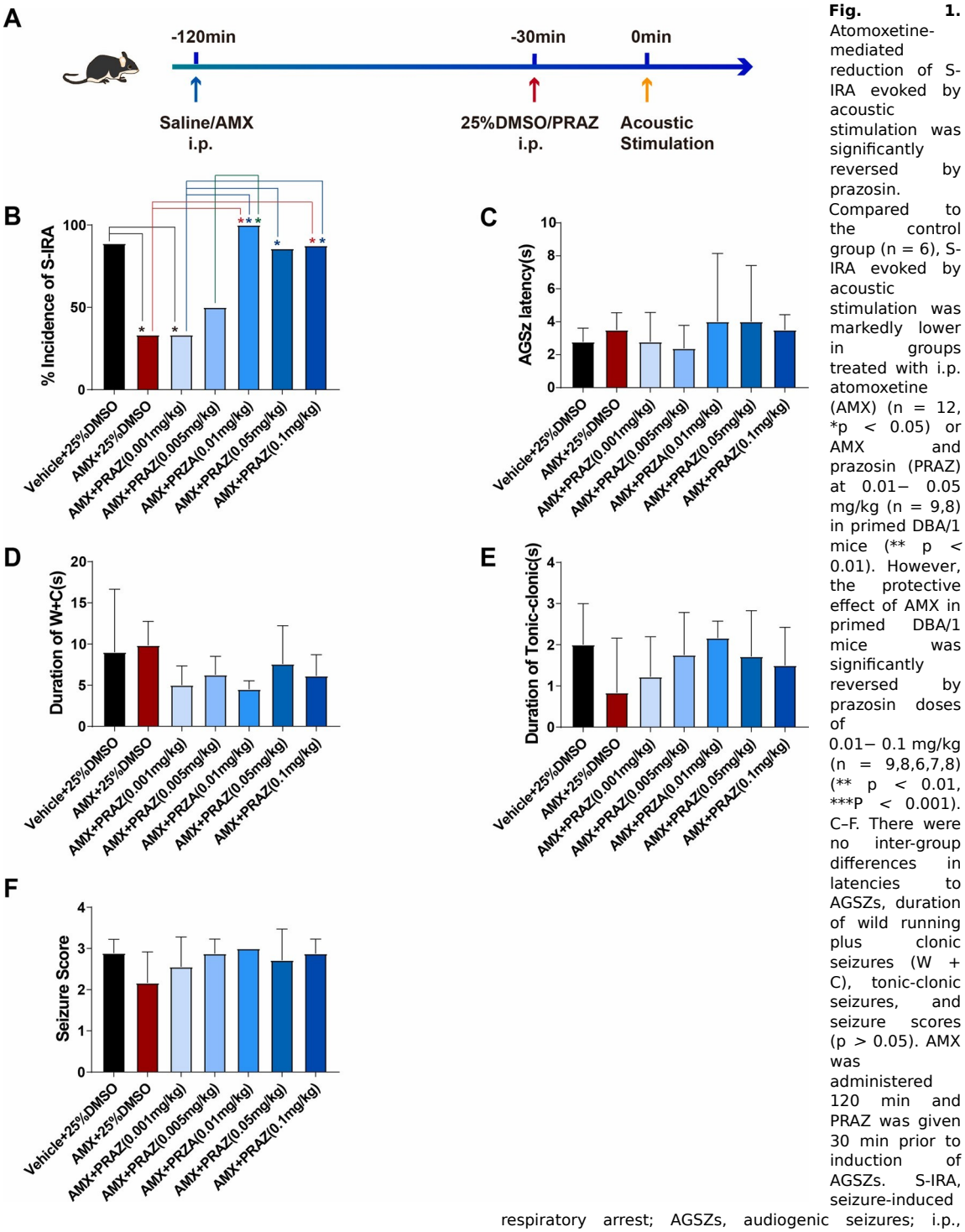


**Fig. 1.** Atomoxetine-mediated reduction of S-IRA evoked by acoustic stimulation was significantly reversed by prazosin. Compared to the control group (n = 6), S-IRA evoked by acoustic stimulation was markedly lower in groups treated with i.p. atomoxetine (AMX) (n = 12, *p < 0.05) or AMX and prazosin (PRAZ) at 0.01− 0.05 mg/kg (n = 9,8) in primed DBA/1 mice (** p < 0.01). However, the protective effect of AMX in primed DBA/1 mice was significantly reversed by prazosin doses of 0.01− 0.1 mg/kg (n = 9,8,6,7,8) (** p < 0.01, ***P < 0.001). C–F. There were no inter-group differences in latencies to AGSZs, duration of wild running plus clonic seizures (W + C), tonic-clonic seizures, and seizure scores (p > 0.05). AMX was administered 120 min and PRAZ was given 30 min prior to induction of AGSZs. S-IRA, seizure-induced respiratory arrest; AGSZs, audiogenic seizures; i.p., intraperitoneal injection; DMSO, dimethyl sulfoxide. (mean ± SEM, n = 8–10).

### *2.7. Measurement the contnet and enzyme activity of tyrosine hydroxylase (TH) in the low brianstem and prosencephalon by ELISA*

Three groups of DBA/1 mice were used for measurements, which included a naive group without acoustic stimulation, a S-IRA group evoked by acoustic stimulation, and a S-IRA group evoked by acoustic stimulation and then resuscitated by small animal respirator. The treated animals were decapitated under anesthesia using an overdose of chloral hydrate (n = 6/group). As we described

previously, the brainstems between bregma− 3 mm and bregma− 9 mm and the prosencephalon were collected after removing excess blood in cold PBS and then immediately frozen in liquid nitrogen and transferred to a − 80 °C refrigerator. Tissue specimens were weighed and then homogenized in ice-cold PBS. Homogenates were centrifuged for 5 min at 5000 *g* to obtain supernatant. Both content and enzyme activity of TH was evaluated by ELISA, respectively. All ELISA procedures were performed following the manufacturer's instructions (Mouse TH, ELISA Kit, YanSheng Biological Technology Co., Ltd, ShangHai, CS1028; CS03271, China). To measure the content of TH, we added samples, standard samples and HRP-labeled antibodies sequentially to the micropore coated with non-phosphorylated tyrosine hydroxylase (TH) antibody. To develop the final detection signal, substrate TMB was added then and converted to blue color under the catalysis of peroxidase. The stop solution changes the color from blue to yellow and the intensity of the color was measured at 450 nm using a spectrophotometer(Molecular Devices, Spectra Max190,NNR06683. For TH content quantitation, the TH ELISA Kit (CS1028) includes a set of calibration standards, by which a standard curve of Optical Density(O.D.) versus TH content(pg/mL)was plotted. The content of TH(pg/mL)in the samples was then determined by comparing the O.D. of the samples to the standard curve. As for the enzyme activity of TH(U/mL), we use the TH ELISA Kit (CS03271) including the phosphorylated TH antibody. The other assay procedures are the same as those for measuring TH content. The enzyme activity of TH(U/mL) in the samples was calculated based on the O.D and measured at 450 nm by a spectrophotometer similarly as described above.

### *2.8. Data analysis*

All data are presented as the mean ± SEM. Statistical analyses were performed using SPSS 23(SPSS Software Inc.,USA). The incidence of S-IRA in different groups was compared using Wilcoxon Signed Rank test. Seizure scores and the latency to AGSZs, the duration of wild running, clonic seizures, tonic-clonic seizures, content and enzyme activity of TH, average EEG spectra were evaluated using the one-way ANOVA tests or Mann Whitney *U* test or Kruskal-Wallis H test or Wilcoxon signed rank test.Paired-samples T test was used for analysis of blood pressure and heart rate from different groups. Statistical significance was determined if $p < 0.05$.

## 3. Results

### *3.1. Atomoxetine-mediated suppression of S-IRA evoked by acoustic stimulation was reversed by prazosin*

Compared to vehicle control, the incidence of S-IRA evoked by acoustic stimulation was significantly reduced by atomoxetine at an IP dose administration of 15 mg/kg ($p = 0.031$). Atomoxetine-mediated suppression of S-IRA was significantly reversed by IP prazosin (0.01 mg/kg prazosin compared with control group, $p = 0.019$; 0.1 mg/kg prazosin compared with control group, $p = 0.044$). There were no significant intergroup differences in latencies to AGSZs, durations of wild running, clonic seizures, tonic-clonic seizures and seizure scores (AGSZs latencies: $p = 0.763$, $F = 6$; durations of wild running: $p = 0.14$, $F = 6$; duration of tonic-clonic seizures: $p = 0.119$, $F = 6$; seizure scores: $p = 0.304$, $F = 6$) (Fig. 1).

### *3.2. The decreased enzyme activity of TH resulting in S-IRA was significantly reversed by mechanical ventilation in lower brainstem*

We examined the content and enzyme activity of TH in the brainstem of DBA/1 mice from 3 groups that included a control group without acoustic stimulation, and two S-IRA groups following acoustic stimulation with and without resuscitation, respectively. Compared to the relative optical density of DBA/1 mice without acoustic stimulation, to which the other two groups were normalized, TH content was not significantly reduced in the brainstem of primed DBA/1 mice ($p = 0.052$; $p = 1.00$, $n = 6$). TH content levels were not significantly different between the S-IRA with and without resuscitation groups ($p = 0.052$, $n = 6$). However, compared with treatment of brainstem, there was a significant reduction of TH content in prosencephalon in the control and S-IRA (rescue) ($p = 0.026$) groups. (Fig. 2.A). Compared with the control treatment of brainstem, TH enzyme activity was significantly decreased in prosencephalon in control ($p = 0.02$), S-IRA ($p = 0.02$) and S-IRA (rescue) ($p = 0.015$) groups ($n = 6$). Compared with the TH enzyme activity in brainstem in the control group, there was a significant reduction in brainstem in S-IRA (rescue) group ($p = 0.04$, $n = 6$). Compared with the TH enzyme activity in prosencephalon in the control group, there was a significant reduction in prosencephalon in S- IRA (rescue) group ($p = 0.02$, $n = 6$). (Fig. 2 B).

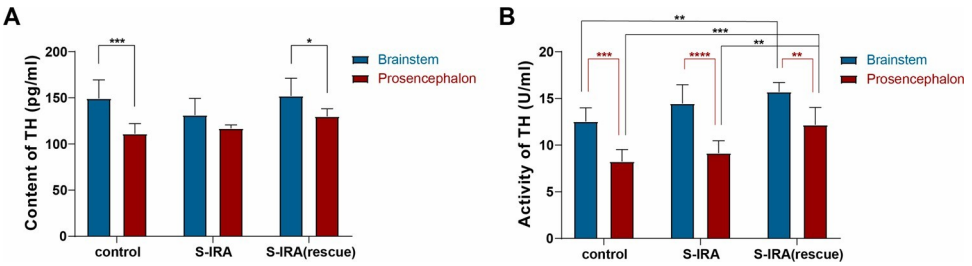


**Fig. 2. Continuous *acoustic stimulation did not change the content of TH but change its activity in brainstem.***

Compared to the control group without acoustic stimulation, there were no significant changes in TH levels in lower brainstem in the S-IRA without resuscitation and S-IRA with resuscitation groups (n = 6) (p > 0.05). There were no significant differences of TH levels in lower brainstem between S-IRA without resuscitation and S-IRA with resuscitation groups (n = 6) (p > 0.05). However, the content of TH in lower brainstem was significantly than in prosencephalon in control and S-IRA (rescue) groups. Compared with the TH activity in brainstem in control group, there was a significant reduction in brainstem in S-IRA (rescue) group. (***P < 0.001, n = 6). Compared with the TH activity in prosencephalon in control group, there was a significant reduction in prosencephalon in S- IRA (rescue) group. (***P < 0.001, n = 6).

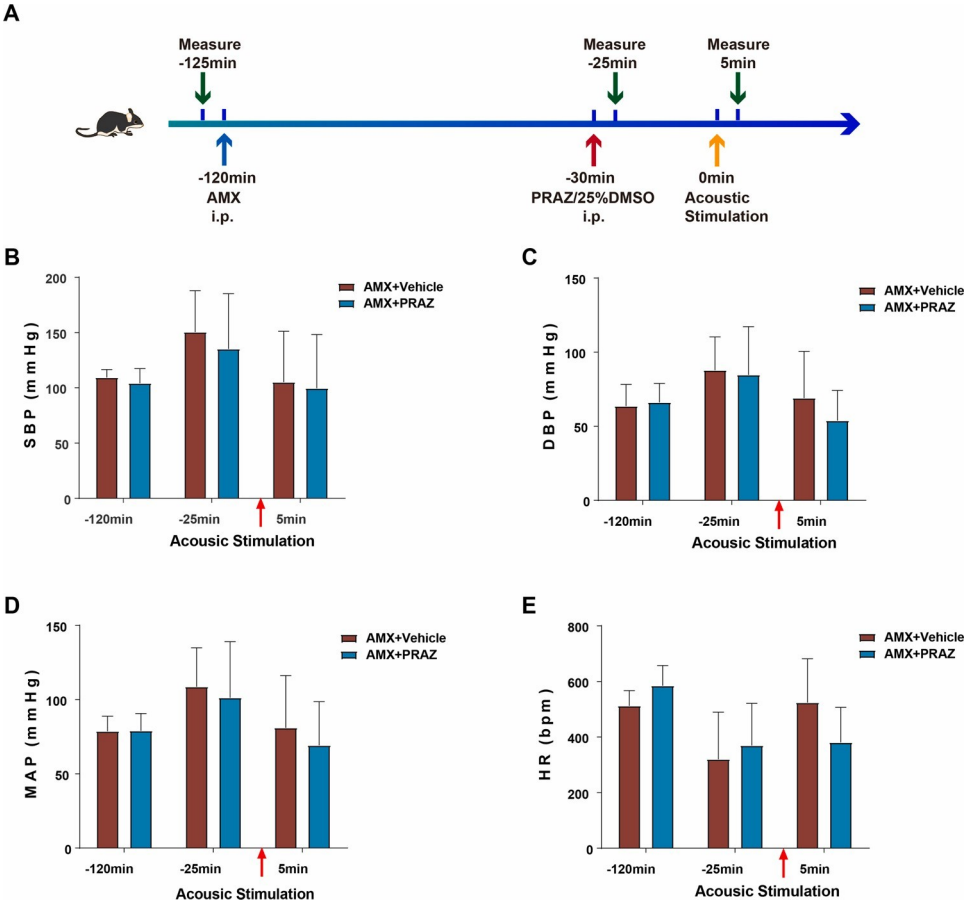


**Fig. 3.** Effects of prazosin on the blood pressure and heart rate. B-E. Compared to the control group (n = 6) in which atomoxetine (AMX) was given i.p. 120 min and 25 % DMSO given i.p. 30 min before and 5 min after acoustic stimulation, there were no significant differences in SBP, DBP, MAP and HR in the group in which AMX was given 120 min prior to acoustic stimulation, and prazosin (PRAZ i.p) (n = 6) 30 min before and 5 min after acoustic stimulation (p > 0.05). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; i.p., intraperitoneal.

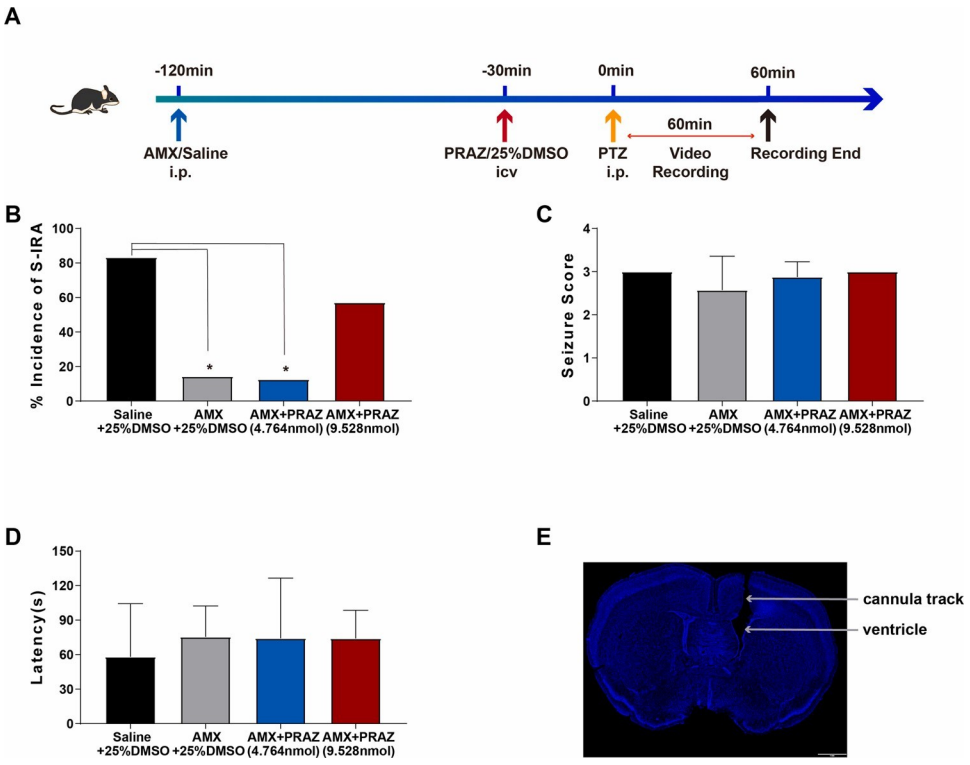


**Fig. 4.** Effect of ICV prazosin on S-IRA evoked by PTZ.
B. Compared to the control group (n = 6) treated with vehicle (IP saline and ICV 25 % DMSO), the incidence of S-IRA evoked by PTZ was suppressed in the group receiving atomoxetine (AMX) i.p. and 25 %DMSO i.c.v., and the group treated with AMX i.p. and ICV prazosin (PRAZ) (4.764 nmol i.c.v.) (n = 5) (**p < 0.01). The suppression of S-IRA by atomoxetine was significantly reversed by a higher dose of
PRAZ (9.528 nmol ICV) (n = 7) (*p < 0.05). C–D. There were no significant differences of seizure scores and latencies among treated groups (p > 0.05). E. A representative histologic section indicating that vehicle or prazosin (PRAZ) were delivered successfully into the ventricle. PTZ, pentylenetetrazole; IP, intraperitoneal; ICV, intracerebroventricular; DMSO, dimethyl sulfoxide.

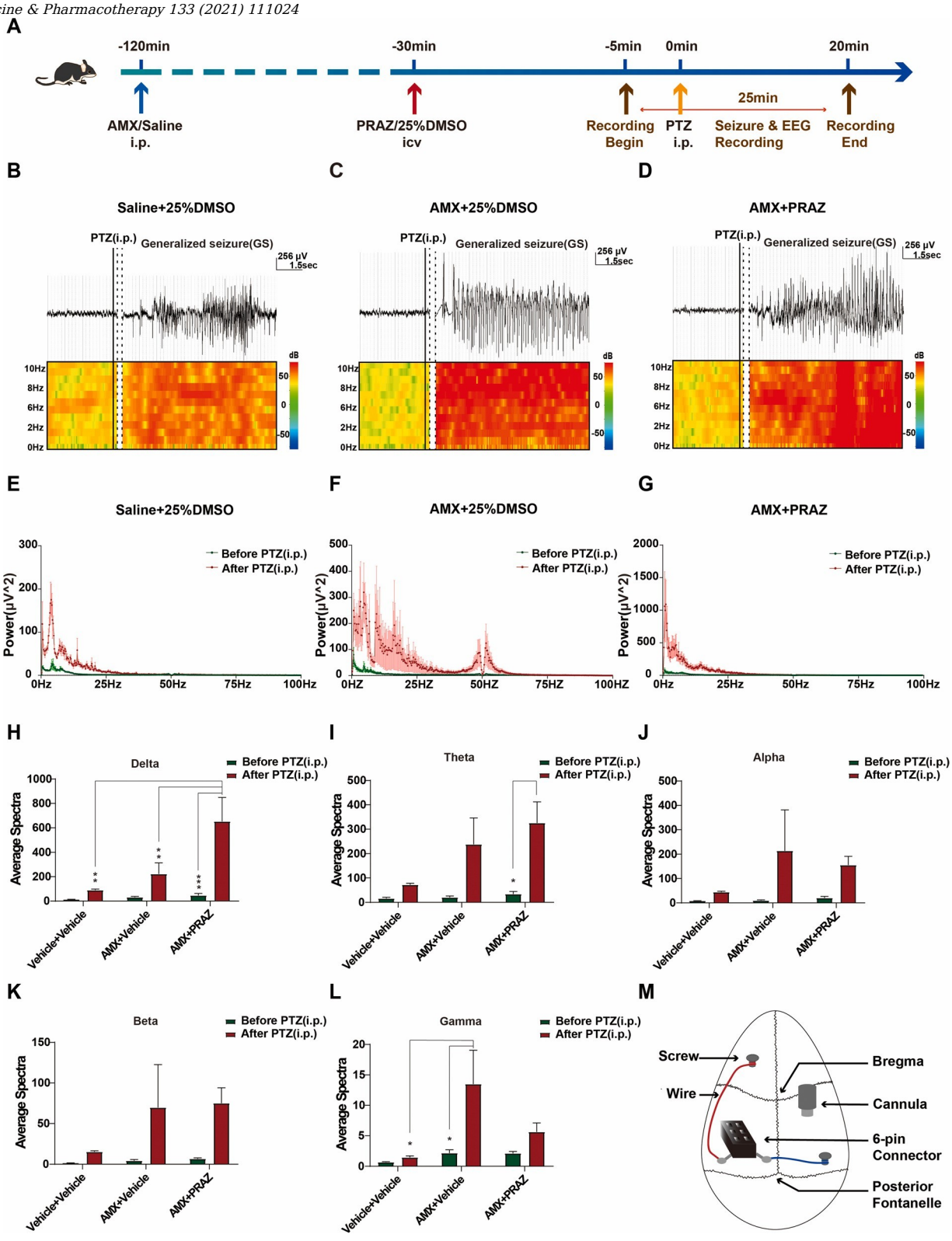


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**Fig. 5.** Effect of ICV prazosin on EEG in the

PTZ-induced SUDEP model.

Sample EEG tracings of PTZ-induced seizures in three3 different treatment groups. A.The timeline precedence of different solvent was to given in the process of measuring recording EEG data. B–D, The spectrograms show the energy spectrum analysis of the EEG above. Changes of power and frequency oscillation peaks in the group treated with atomoxetine (AMX) 120 min and 25 % DMSO i.c.v. 15 min prior to PTZ respectively, were similar to the control group. D and G. Changes of voltage amplitude and frequency oscillation peaks in the group treated with AMX + prazosin (PRAZ) (9.528 nmol, i.c.v.) were administered 120 min and 15 min prior to PTZ respectively, were not decreased compared to the control and AMX + DMSO groups. E–G. Changes of voltage amplitude peak (power) frequency oscillation peaks increased after PTZ injection in the group in which saline i.p. and 25 % DMSO i.c.v. (n = 3) were administered 120 min and 15 min prior to PTZ, respectively, as controls. H–L. Compared to the Vehicle + Vehicle and AMX + Vehicle groups, the average delta band spectra in the AMX + PRAZ group significantly increased (**p < 0.01). I. The average theta band spectra in the AMX + PRAZ group increased after PTZ (IP) (**P < 0.01). J. There were no significant intergroup changes of average alpha band spectra. K. There were no significant intergroup differences of average beta band spectra. L. Compared to the vehicle + vehicle and AMX + vehicle groups, the average spectra of gamma band in the AMX + i.p. vehicle group increased after PTZ administration. (***P < 0.001). M. a representative illustration for the implantation of EEG electrode and ICV cannula in DBA/1 mice. PTZ, pentylenetetrazole; DMSO, dimethyl sulfoxide; i.p., intraperitoneal; i.c.v., intracerebrovascular. H–L. The green line indicated EEG data obtained before PTZ injection, and the red one obtained after PTZ injection.

### *3.3. Effects of prazosin on blood pressure and heart rate*

Given that prazosin can decrease peripheral blood pressures, affect heart rate, and reduce cerebral perfusion pressure, which may cause S-IRA in our model, we measured changes of SBP, DBP, MAP and HR 5 min after acoustic stimulation. There were no significant differences of SBP, DBP, MAP and HR between the vehicle control group in which IP atomoxetine (15 mg/kg) and 25 % DMSO were given 120 min and 30 min prior to acoustic stimulation, respectively; and the experimental group in which IP atomoxetine (15 mg/kg) and prazosin were given 120 min and 30 min prior to acoustic stimulation, respectively ($p > 0.05$, n = 6/group) (Fig. 3).

### *3.4. Effects of ICV prazosin administration on atomoxetine-mediated suppression of S-IRA evoked by PTZ*

To differentiate whether there is possible differential effects of prazosin on atomoxetine-mediated suppression of S-IRA evoked by different epileptogenic stimuli, we further chose an established SUDEP model using PTZ to induce S-IRA. Compared to the vehicle control group, PTZ-induced S-IRA was markedly suppressed by atomoxetine ($p = 0.017$). Compared to the control group, low-dose prazosin (atomoxetine, 15 mg/ kg, i.p, prazosin, 4.764 nmol, icv) significantly reduced the incidence of S-IRA induced by PTZ ($p = 0.011$), which suggested that the low incidence of S-IRA by atomoxetine was not significantly reversed by low-dose prazosin. However, compared to the vehicle control group, the high-dose prazosin (atomoxetine, 15 mg/kg, i.p, prazosin, 9.528 nmol, icv) did not significantly reduce the incidence of S-IRA ($p = 0.077$), instead, which indicated that atomoxetine-mediated suppression of S- IRA was significantly reversed by a higher dose of prazosin. There were no significant inter-group differences in seizure scores between different groups ($p = 0.274$). (Fig. 4)

### *3.5. ICV atomoxetine and prazosin administration did not attenuate PTZ- induced EEG changes*

We chose the PTZ model to evaluate the effects of prazosin on EEG activity. The EEG voltage amplitude peak (power) was significantly increased after PTZ injection in all three groups (Fig. 5). Meanwhile, the low frequency oscillation peak appeared before PTZ injection, and high-frequency oscillations peaks occurred after PTZ injection in the three groups (Fig. 5, B–G). Compared with those observed before PTZ injection, the average spectra of delta, theta and gamma bands increased after PTZ administration. Compared to the vehicle + vehicle and atomoxetine + vehicle groups, the average delta band spectra in the atomoxetine + prazosin group significantly increased (**p < 0.01). The average theta band spectra in the atomoxetine + prazosin group increased after PTZ (IP) (**P < 0.01). These results showed that atomoxetine reduced the incidence of S-IRA without suppressing seizure and EEG activity. (Fig. 5, H–L) (Tables 1 and 2 ).

## 4. Discussion

Although initial progress has been made to identify the causes of SUDEP, the pathogenesis of SUDEP remains still unexplored [4–6]. Herein, our results provide new insights into the role of central norepinephrinergic neurotransmission to elucidate the cause of in SUDEP in the DBA/1 mice SUDEP model. Our data showed that the atomoxetine-mediated suppression of S-IRA can be significantly reversed by administering prazosin via IP and ICV injection in AGS DBA/1 and PTZ mice SUDEP model, respectively (Fig. 6).

Out of consideration for NEα-1Rs distributing both in peripheral and central blood vessel and nervous system, based on the observation already made by peripheral administration of prazosin via IP, we further explore if ICV administration of prazosin can reverse the suppression of

**Table 1**
Summary of experimental groups of DBA/1 mice.

| Figure | Experimental groups | Numbers of mice |
|---|---|---|
| Fig.1 | Vehicle+25 %DMSO | 9 |
| | AMX+25 %DMSO | 6 |
| | AMX + PRAZ(0.001 mg/kg) | 9 |
| | AMX + PRAZ(0.005 mg/kg) | 8 |

| | | |
|---|---|---|
| | AMX + PRZA(0.01 mg/kg) | 6 |
| | AMX + PRAZ(0.05 mg/kg) | 7 |
| | AMX + PRAZ(0.1 mg/kg) | 8 |
| | Control S- | 6 |
| Fig.2 | IRA | 6 |
| | S-IRA (rescue) | 6 |
| Fig3 | AMX + Vehicle | 6 |
| | AMX + PRAZ | 6 |
| | Vehicle+25 %DMSO | 9 |
| | AMX+25 %DMSO | 6 |
| Fig.4 | AMX + PRAZ(4.764 nmol) | 9 |
| | AMX + PRAZ(9.528 nmol) | 8 |
| | Saline+25 %DMSO | 3 |
| Fig.5 | AMX+25 %DMSO | 5 |
| | AM | |

**Table 2**
Statistical Analysis.

| Figure | Compa |
|---|---|
| | Inciden |
| | AGSz la |
| | Duratic |
| Fig.1 | |
| | Duratic |
| | Seizure |
| Fig.2 | Conten |
| | Enzym |
| | SBP |
| | DBP |
| Fig.3 | MAP |
| | HR |
| | Inciden |
| | Seizure |
| Fig.4 | |
| | Latency(s) |
| Fig.5 | Average Spectra |

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| | Kruskal-Wallis H test |
| | One-Way ANOVA |

reduction of S-IRA was 1000 times higher than that used in our DBA/1 mice SUDEP model [18]. The higher prazosin dose may produce cardiovascular or other non-CNS side-effects that will reverse the effect of atomoxetine on S-IRA in the MES model. Consequently, its reversal by high-dose prazosin lacks specificity for understanding S-IRA. In addition, as the authors emphasized, the MES model of epilepsy has low fidelity to recapitulate human disease, which precludes the design of clinically relevant controls [14]. Still, there is some limitations in our findings such as in our model atomoxetine may target other monoaminergic neurons to modulate S-IRA, the possibility of which can not be excluded. Nevertheless, our findings of the role of NEα-1R in modulating S-IRA supported by those from the MES model will advance the elucidation of the pathogenesis of S-IRA and SUDEP, and help design therapeutic strategies to prevent SUDEP.

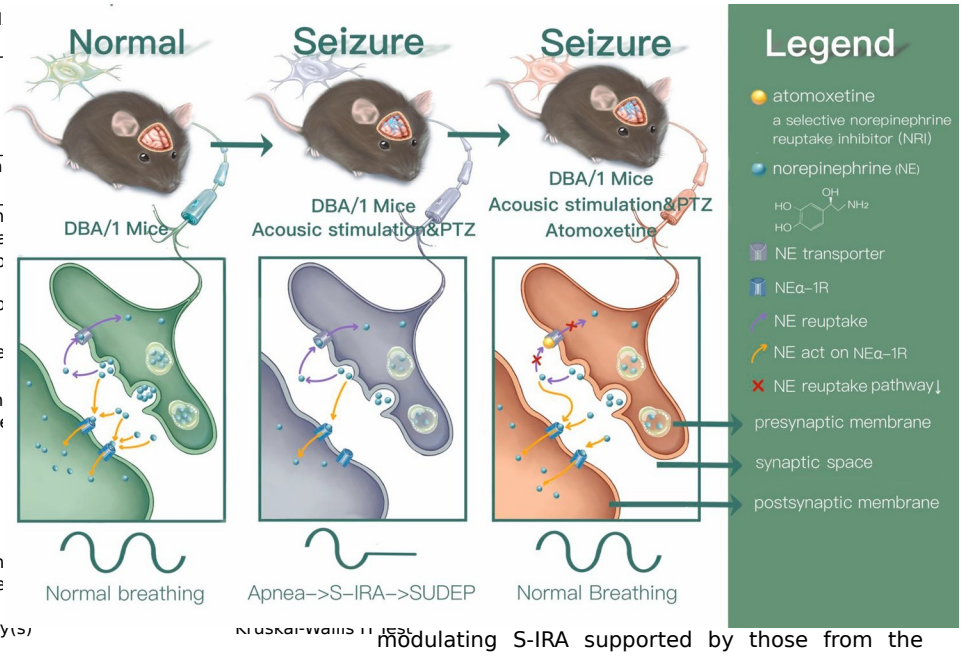


**Fig. 6.** Mechanism of atomoxetine-mediated reduction of S-IRA was reversed by prazosin in the central nervous system. Norepinephrine (NE); NE α-1 receptor (NEα-1R); NE transporter; Atomoxetine (a selective norepinephrine reuptake); Prazosin; NE reuptake; NE reuptake pathway;
presynaptic membrane; synaptic space.

S-IRA by atomoxetine via NEα-1R in the central pathway. Similar reversal effect was observed, in agreement with that by IP administration. This suggest that prazosin may target central NEα-1R in brain to reverse the suppression of S-IRA by atomoxetine and the disclosure of exact functional region in brain where targeted NEα-1Rs are located needs to be explored.

In our models, atomoxetine-mediated suppression of S-IRA was reversed by low-dose prazosin. This is consistent with our previous and other investigators' findings that atomoxetine reduced the incidence of S-IRA in a DBA/1 murine and other SUDEP models. Also the NEα-1R's potential role in modulating S-IRA and SUDEP was explored in lmx1bf/ f/p mice with deletion of 5-HT neurons through a maximal electroshock (MES) model, although potential problems in their studies need to be further considered [9,110,18]. For example, in the MES model, the dose of prazosin that reversed atomoxetine-mediated

Further, we tested whether atomoxetine-mediated suppression of S- IRA depends on enhanced NE bioavailability in the synaptic space by TH enzyme activity or TH content changes in the lower brainstem. Our adoption of two complementary murine models benefited our study. First, we used a murine model of SUDEP evoked by acoustic stimulation to test the role of NEα-1R in modulating S-IRA as its own control. Second, we chose a model of SUDEP evoked by PTZ that recapitulates human generalized seizures to test the role of NEα-1R in modulating S- IRA. Furthermore, we explored the reversal effects of prazosin from behavioral and electrophysiologic perspectives as discussed below. Importantly, we provide the first changes of brainstem TH content and enzyme activity following S-IRA, which suggests that atomoxetine- mediated inhibition of S-IRA depends on enhanced bioavailability of NE in the synaptic space and TH enzyme activity rather than TH content in the lower brainstem,

demonstrating that the TH enzyme activity in lower brainstem plays a key role in mediating S-IRA.

To exclude the cardiovascular effects of prazosin on the reversal of atomoxetine-mediated suppression of S-IRA, we measured the changes of SBP, DBP, MAP and HR among different treatment groups. Prazosin used within the our dose range did not significantly change SBP, DBP, MAP and HR. However, other studies have shown that arrhythmia can cause SUDEP [19,20], and the potential role of arrhythmia was not ruled out in our model. Prazosin is commonly used to treat hypertension, and is potentially used in epilepsy patients at risk for SUDEP. However, our results suggest that alternative anti-hypertensive agents should be reconsidered for epilepsy patients. In addition, we previously reported that repeated generalized seizures can produce calcified cardiac lesions in DBA/1 mice [21], it is essential for us to clarify the role of the cardiovascular system in SUDEP in the near future. It should be mentioned although the reversal of atomoxetine-mediated reduction of S-IRA by prazosin is independent of cardiovascular function, due to the limitation of models, we could not directly monitor the changes of blood pressure due to technical challenge of implanting the tube into internal carotid artery of DBA/1 mice to timely and immediately observe it's changes.

Our data showed that atomoxetine-mediated reduction of S-IRA was reversed by prazosin without suppressing seizure behavior and EEG activity. For one thing, we assessed the seizure score and measured the AGSZ latency and duration of clonic and/or tonic-clonic seizures to analyze seizure behavior. There were no significant intergroup differences of these parameters. For another, atomoxetine reduced the incidence of S-IRA without suppressing EEG delta, theta and gamma activity. In addition, in our model, taking account into the different kinetics of atomoxetine and prazosin, respectively [22,23], we chose to give prazosin 30 min before administration of atomoxetine and the data showed atomoxetine-mediated suppression of S-IRA was reversed by prazosin in a dose-dependent manner with ceiling effects, suggesting that the central NEα-1R is a rational therapeutic target for the prevention of SUDEP. However, a recent study showed that the suppressant effects of S-IRA by atomoxetine was not significantly reversed by prazosin (1 mg/kg,i.p.) given before atomoxetine in the same model [24]. By contrast, in our study, the suppression effects of S-IRA by atomoxetine was markedly reversed by prazosin at the dose 0.01 mg/kg (i.p.). Analyzing the cause for this, the timing of administration with prazosin may be a critical influence factor. Furthermore, in their study, the lower incidence of S-IRA by atomoxetine was significantly reversed by yohimbine, a NEa-2 receptor antagonist [24]. Of course, it is possible that the NEa-1 and NEa-2 receptor mediate SUDEP by mutual interaction, which will be explored in the near future. Considering NEα-1R locating in pre-Botzinger complex (preB¨ otC) to mediate respiration ¨ function [12–14], we speculate that atomoxetine-mediated suppression of S-IRA was reversed by prazosin acting in preBotC in our models. ¨ However, it need to be further tested in coming experiments.

A limitation of our study was that we did not identify the NEα-1R- mediated neural circuits involved in S-IRA, just as we previously had not characterized the neural circuits that suppress S-IRA evoked by either acoustic stimulation or PTZ injection in DBA/1 mice by activating serotonergic neurotransmission neurons in the dorsal raphe nucleus using optogenetic technology [8]. Additionally, we did not establish a model of ADHD and epilepsy comorbidity to test whether the role of atomoxetine in suppression of SUDEP in ADHD and epilepsy co-morbidity. Nevertheless, our findings suggest that epilepsy patients receiving atomoxetine for ADHD may be receiving an additional benefit of reduced risk of SUDEP, and that the use of prazosin as an anti-hypertensive agent should be avoided in this patient population.

## 5. Conclusion

Our results provide new insights into the pathogenesis of SUDEP and emphasized the importance of enhancing the central synthesis of NE and norepinephrinergic neurotransmission in brain for prevention of SUDEP. The study suggested that atomoxetine is a promising candidate for translational research to prevent SUDEP in patients with ADHD and epilepsy co-morbidity. Essentially, targeting the central NEα-1R may provide the rational therapeutic strategies to prevent SUDEP or reduce the incidence of SUDEP.

## Funding

The work was supported by the National Natural Science Foundation of China (Grant.NO: 81771403, 81974205); by the Natural Science Foundation of Zhejiang Province (LZ20H090001); by the Program of New Century 131 outstanding young talent plan top-level of Hang Zhou to HHZ; and by the National Natural Science Foundation of China (Grant.NO: 81,771,138) to Han Lu and by the National Natural Science Foundation of China (Grant.NO: 82,001,379) to HTZ. This manuscript has been released as a pre-print at BioRxiv [25].

## Declaration of Competing Interest

All authors declare no competing interests. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this study is in accordance with those guidelines.We are grateful for Yi Shen and Yu Dong Zhou for the experimental design and for YingHui Zhang for the language editing.

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